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Pilidiella crousii sp. nov. from the northern Western Ghats, India

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Abstract — The coelomycete genus *Pilidiella* (*Schizoparmaceae, Diaporthales*) was recently revised to include species with hyaline to pale brown conidia in contrast to the dark brown conidia of *Coniella*. The present paper describes a new species of *Pilidiella, P. crousii*, based on a molecular phylogenetic analysis of the ITS nrDNA and its unique conidial morphology. *Pilidiella crousii* is associated with severe fruit drop of *Terminalia chebula* (*Combretaceae*) in natural forests of Mahabaleshwar in the Western Ghats of India.

Key words — anamorphic fungi, plant pathogen, *Schizoparme*

Introduction

The exploration of microfungal diversity of the Western Ghats, which is one of the biodiversity hot spots of the world, is the prime goal of the National Fungal Culture Collection of India (NFCCI). The NFCCI aims to explore fungi from this region for their biotechnological and bioprospecting potential (Rajeshkumar et al. 2010; Singh et al. 2009). During July 2010 a routine survey was conducted to explore the microfungal diversity in the natural forests of Mahabaleshwar, situated in the northern part of the Western Ghats, India, at 17°58'N and 73°43'E. Discovered during this survey, an interesting species of *Pilidiella* Petr. & Syd. was found on fallen fruits of *Terminalia chebula,* associated with severe fruit infection followed by fruit drop. The aim of the present study is to identify the species of *Pilidiella* associated with fruit drop of *Terminalia* in India.

Materials & methods

Isolates and morphology — Conidiomata of the fungus were directly isolated from the surface of fallen fruits and observed under a Nikon Binocular stereo microscope

(Model SMZ – 1500 with Digi-CAM, Japan). Single conidial cultures were established on 2% Potato Dextrose Agar plates (PDA; Crous et al. 2009). For morphotaxonomic studies and photomicrographs an Olympus CX-41 (Japan) microscope was used. Conidia and conidiomata were mounted in lactic acid cotton blue and measured using an ocular micrometer, with 30 observations per structure. Colony characteristics in culture were studied on different media, viz. 2% Malt Extract Agar (MEA), Potato Carrot Agar (PCA), and PDA (Crous et al. 2009). Herbarium specimens were deposited in Ajrekar Mycological Herbarium (AMH); the culture was accessioned and preserved in the National Fungal Culture Collection of India (WDCM-932), Agharkar Research Institute, Pune, India.

Polymerase chain reaction and sequencing — Total DNA was extracted from cultures grown on PDA plates for two weeks at 25 °C, using a DNA extraction kit as per the manufacturer's instructions (MP Biomedicals GmbH, Germany). Fragments containing the region encoding the ITS 1-5.8S-ITS 2 were amplified using primer pairs ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT- AAC AAG G-3') (White et al. 1990). PCR was performed in a 25 µl reaction using 2 µl of template DNA (10 ng - 25 ng), 0.5 U of Taq DNA polymerase (Genei, Bangalore, India), 2.5 µl of 10X Taq DNA polymerase buffer, 0.5 µl of 200 µM of each dNTPs (Genei, Bangalore, India), 0.5 µl of 10 pmol primer, H_2O (Sterile Ultra Pure Water, Sigma) qsp 25 µl. Amplification was performed on an Eppendorf Mastercycler AG using the following parameters: 5 min step at 95 °C, followed by 30 cycles of 1 min at 95 °C, 30s at 56 °C and 1 min at 72 °C for ITS region amplification, then a final 7 min extension step at 72 °C. The PCR products were purified with Axygen PCR cleanup kit (Axygen Scientific Inc, CA, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were run on an ABI 3100 automated DNA sequencer (Applied Biosystems, USA).

Sequence alignment and phylogenetic analysis — Sequence alignment of partial ITS1-5.8S-ITS2 of *P. crousii* was performed manually using the text editor option of the software Molecular Evolutionary Genetics Analysis (MEGA) software v4.0. (Tamura et al. 2007). The manually edited sequences of NFCCI 2213 were deposited in the NCBI sequence nucleotide database (HQ264189). They were also subjected to a BLAST search. The partial ITS sequences were aligned using Clustal W together with the homologous regions of ITS of closely related species of *Pilidiella* and *Schizoparme* Shear. For ITS, the matrix was analyzed with the Maximum Parsimony method using the Tamura model (Tamura et al. 2007) to calculate the sequence divergence, and the bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option).

Results

DNA phylogeny — For the phylogenetic analysis of the genus *Pilidiella*, the alignment of 557 bp of the ITS sequence data included 450 positions in the final data set. The analysis of ITS1–5.8S–ITS2 sequence presented here (Fig. 1) reveals a significant association of *Schizoparme* and its anamorphic genus *Pilidiella*. The phylogenetic analysis based on ITS sequences resulted in four

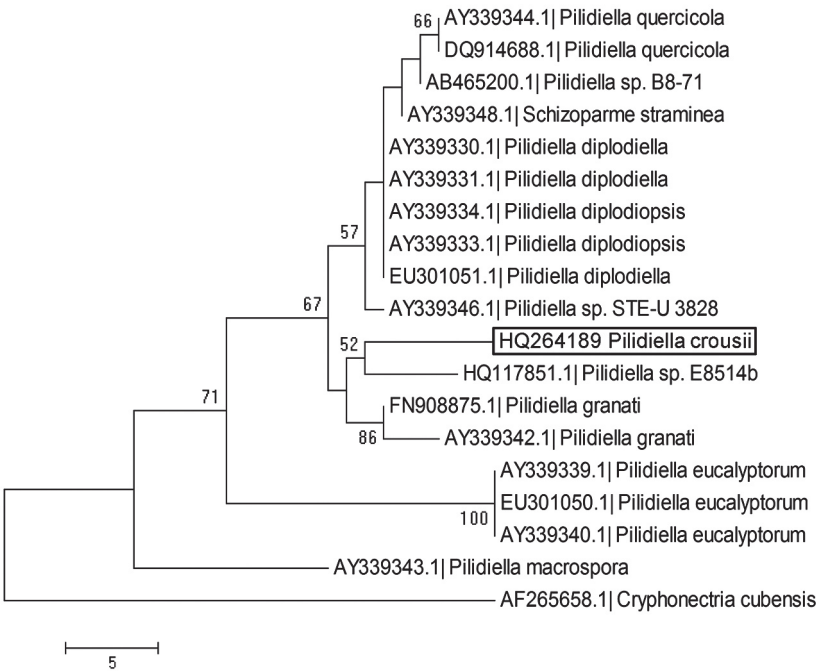

Fig. 1. Phylogenetic tree based on aligned internal transcribed spacer sequences of *Schizoparme* and its anamorphic species of *Pilidiella*. The consistency index is (0.660000), the retention index is (0.821053), and the composite index is 0.674127 for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The MP tree was obtained using the Close-Neighbour-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale; with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence.

major clades, *P. diplodiella* (Speg.) Crous & Van Niekerk, *P. diplodiopsis* Crous & Van Niekerk, *P. quercicola* (Oudem.) Petr. and *S. straminea* Shear clustered together and formed a major group. *Pilidiella eucalyptorum* Crous & M.J. Wingf. formed a unique clade (100% similarity). *P. macrospora* (71% similarity) clustered as a unique clade. The new species, *P. crousii,* clustered with *Pilidiella* sp. (HQ 117851.1) (52% similarity), which was sister to *P. granati* (Sacc.) Aa. *Cryphonectria cubensis* (Bruner) Hodges was chosen as outgroup, as it belongs to the *Cryphonectria–Endothelia* complex that is allied to the *Schizoparme* complex (Castlebury et al. 2002).

Taxonomy — Based on a DNA comparison with other species currently in GenBank (Fig. 1), and its unique morphological characters (Fig. 2), such as the highly variable conidial shape, size and length-width ratio, the species of *Pilidiella* from *Terminalia* can be distinguished from the other taxa in this genus, and hence is described as new to science.

Pilidiella crousii Rajeshkumar, S.K. Singh & Hepat, **sp. nov.** Fig. 2

MycoBank MB 518897

In fructis. Pycnidia globosa vel subglobosa, 124–280 × 115–190µm, laeves, pariete multitunico ex 2–4 stratis et textura angulari. Conidiophora 15–25 × 2.5–3 µm, simplicia vel ramose. Cellulae conidiogenae 6–12 × 2–3µm, simplices, laeves. Conidia primo hyalinia, deinde pallida vel modice brunnea, forma et amplitudine valde variabilibus, laevia, ellipsoidea vel anguste ellipsoidea, apice acute rotundato vel subobtuso, basi truncata, (6–)7–12(–13.5) × (2.5–)3–5 µm (plus minusve 9.5 × 4.0 µm), ratione longitudinis/ latitudinis = 2.2–2.3.

Holotype: India, Mahabaleshwar, Western Ghats, Maharashtra, on fallen fruits of *Terminalia chebula* (*Combretaceae*) July 2010, K.C. Rajeshkumar (AMH 9406; ex-type culture NFCCI 2213.)

Etymology: '*crousii*' named in honour of Prof. dr. Pedro W. Crous, Director, Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands, for his scientific contribution to this fungal group.

On fruits, forming pustule-like structures erupting through pericarp, scattered over fruit surface; conidiomata aggregated in a dark reddish brown mass. Conidiomata pycnidial, globose to sub-globose, 124–280 × 115–190 µm, smooth; wall of textura angularis, initially hyaline, becoming pale to medium brown, consisting of 2–4 layers. Conidiophores developing from a basal or central, hyaline cushion of cells, simple or branched, densely packed, encased in mucous, 15–25 × 2.5–3 µm. Conidiogenous cells simple, smooth, 6–12 × 2–3µm. Conidia initially hyaline, becoming pale to medium brown when mature, smooth, highly variable in shape and size, straight, occasionally slightly curved, ellipsoidal to narrowly ellipsoidal, with subobtuse apex and base or truncate base in a few conidia, but acutely rounded apices also present in others, bi- to multiguttulate, (6–)7–12(–13.5) × (2.5–)3–5 µm (mean 9.5 × 4.0 µm), length-width ratio = 2.2–2.3.

Teleomorph: Unknown; no sexual state or fungus resembling *Schizoparme* was present on any specimen examined.

Colonies on PDA fast growing, 79 mm diam after 7 days and 90 mm diam after 10 days, white initially, later turning pale brown to dark brown, floccose, reverse pale to dark brown. Colonies on MEA fast growing, 80 mm diam after 7 days, initially white, brownish when mature, margin irregular, reverse dark gray-brown. Colonies on PCA fast growing, 60 mm diam after 7 days, pale brown margin regular, colonies reverse dark brown.

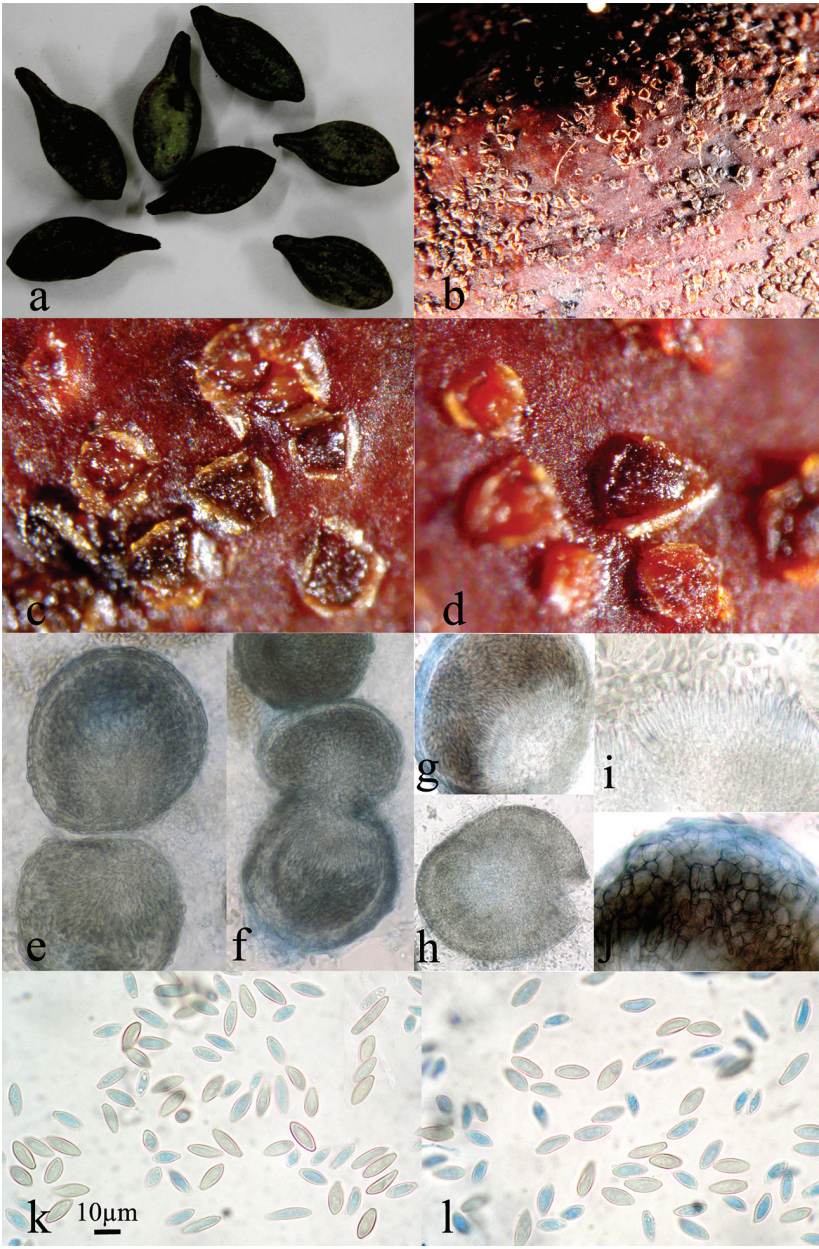

Fig. 2. *Pilidiella crousii* (holotype). a. Fallen fruits of *Terminalia chebula*. b–d. Conidiomata on fruits. e–h. Pycnidia in culture. i. Conidiophores developing from basal pad of tissue. j. Pycnidial wall. k–l. Variable shaped conidia. Bars: k, l = 10 µm.

Discussion

Petrak & Sydow (1927) established the genus *Pilidiella* with hyaline to pale brown conidia, differentiating it from *Coniella* Höhn., which has dark brown conidia. Later, von Arx (1957, 1972) also supported the differentiation of these genera based on their conidial pigmentation. However, Sutton (1980) and Nag Raj (1993) treated *Pilidiella* as a synonym of *Coniella*.

Castlebury et al. (2002) determined that *Pilidiella* with its teleomorph *Schizoparme* formed a distinct lineage within the *Diaporthales*, representing a genus separate from *Coniella*. Later, Van Niekerk et al. (2004) conducted an extensive study of the group, including a thorough molecular analysis based on ITS, EF1-α and LSU gene sequence data. Based on all three data sets, they confirmed the separation of *Pilidiella* (typified by *P. quercicola* (Oudem.) Petr. [= *P. castaneicola*]) from *Coniella* (typified by *C. pulchella* Höhn. [= *C. fragariae*]). *Pilidiella* is characterized by having species with hyaline to pale brown conidia with a length-width ratio >1.5 in contrast to dark brown conidia of *Coniella* with length-width ratio ≤1.5.

Samuels et al. (1993) linked several species of *Coniella* to species of *Schizoparme*, which they regarded as a member of *Diaporthales* (*Melanconidaceae*). However, Castlebury et al. (2002) suggested that the *Schizoparme*-complex is representative of an undescribed family in the *Diaporthales*, with *Coniella* and *Schizoparme–Pilidiella* as separate genera. In a further study, Rossman et al. (2007) established *Schizoparmaceae* Rossman (type genus: *Schizoparme*) to accommodate these genera, with diagnostic characteristics "*Ascomata fusca vel nigra, collapsa, erumpentia, superficialentia. Asci annulo apicali distincto praediti, ad maturitatem separati, paraphyses nullae. Ascosporae non septatae*". The pycnidial anamorphs of this family are *Coniella* and *Pilidiella*. The type species of *Schizoparme* is *S. straminea*, which is linked to the anamorph *Pilidiella castaneicola* (Ellis & Everh.) Arx. In contrast with the *Schizoparme* teleomorphs associated with *Pilidiella*, no teleomorph has thus far been reported for *Coniella* (Rossman et al. 2007).

In India, Sharma et al. (1985) studied the genus *Coniella* from *Eucalyptus* plantations in Kerala State. They reported *C. castaneicola* (Ellis & Everh.) B. Sutton and *C. fragariae* (Oudem.) B. Sutton causing a leaf spot disease in *E. grandis* and *E. tereticornis*. Van Niekerk et al. (2004) pointed out that the isolates from *Eucalyptus* treated as *C. fragariae* should be recognized as *P. eucalyptorum*. *Coniella granati* (Sacc.) Petr. & Syd. was reported as a saprobe on leaf litter of *Tectona grandis* (Mary & Sankaran 1991; Sankaran 1994). Rajeshkumar (2007) explored the pathogenic microfungal diversity in natural forests in the Kerala part of the Western Ghats, and reported four species of *Coniella*: *C. australiensis* Petr. on *Macaranga peltata*, *C. minima* B. Sutton & Thaung on *Garcinia gummi-gutta*, *C. petrakii* B. Sutton on *Careya arborea* and *Syzygium caryophyllatum*, and *C. fragariae* on more than 10 different hosts plants in natural forests.

Coniella spp. have been reported as important foliar pathogens of *Terminalia* spp. in India. *Coniella terminaliae* (Firdousi et al. 1994) was identified as a new species causing necrotic spots on *Terminalia tomentosa* (IMI 823384) from the forests of Gopalpura (Sagar), Madhya Pradesh. This species is close to *C. fragariae* in shape and colour of its pycnidia and conidia, but its conidia are smaller (2—8 × 3—5 µm) and globose to subglobose in shape. Rajeshkumar (2007) also recorded foliar diseases caused by *Coniella fragariae* on *Terminalia chebula* and *T. paniculata* in natural forests of Kerala. *Coniella macrospora* (van der Aa 1983) is the only species under this genus so far recorded on *Terminalia ivorensis* from the Ivory Coast, but its conidia are much larger, (18.3–)25–29 (–32.5) × (13–)16–20(–21.5) µm.

In the present study, *Pilidiella crousii* is proposed as a new species based on the ITS sequence analysis (Fig. 1) and highly variable shape and size of its conidia (Fig. 2). The morphological characteristics of *P. granati* differ from those of the new species. In *P. granati* the conidia are ellipsoidal, 9–16 µm long, and length- width ratio is 1.9. In *P. crousii*, the conidia are ellipsoidal or narrowly ellipsoidal, (6–)7–12(–13.5) µm long, with a length -width ratio of 2.2–2.3. The conidial length-width ratio of *P. crousii* is close to that of *P. diplodiella,* but the conidia in *P. diplodiella* are longer. Also the ITS sequence analysis clearly differentiates these two species.

Appendix

In their treatment of *Pilidiella* and *Coniella*, Van Niekerk et al. (2004) referred to *Coniella macrospora* as "*Pilidiella macrospora*", but did not make a valid combination. This combination is proposed here:

Pilidiella macrospora (Aa) Crous & Van Niekerk, comb. nov.

MycoBank MB 517391

Basionym: *Coniella macrospora* Aa, Proc. Kon. Ned. Akad. Wetensch., C 86: 121. 1983.

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